PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A01K 67/027, C12N 5/16 // C07K 14/71

A1

(11) International Publication Number: **WO 99/62332**

(43) International Publication Date: 9 December 1999 (09.12.99)

(21) International Application Number: PCT/FI99/00464

(22) International Filing Date: 27 May 1999 (27.05.99)

(30) Priority Data:
981217 29 May 1998 (29.05.98) FI

(71)(72) Applicants and Inventors: AIRAKSINEN, Matti, Sakari [FI/FI]; Koetilankuja 2 H 58, FIN–00710 Helsinki (FI). SAARMA, Mart [FI/FI]; Kulosaaren puistotie 38 A 4, FIN–00570 Helsinki (FI).

(74) Agent: BORENIUS & CO. OY AB; Kansakoulukuja 3, FIN–00100 Helsinki (FI).

(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: TRANSGENIC ANIMALS LACKING A FUNCTIONAL GFRα2 RECEPTOR AS WELL AS METHODS FOR THEIR PRODUCTION AND USE

(57) Abstract

The present invention is related to cell–lines and transgenic non–human animals lacking a functional GFRα2 receptor. Methods for production of said cell–lines and transgenic animals as well as their use are also disclosed. The transgenic non–human animals are useful as model for developing therapies, studying and diagnosing growth related disorders, human sensory and autonomic nervous system disorders involving gut dysfunction and disorders in the specific peripheral nervous system (PNS), including autonomic and sensory functions as well as in the central nervous system (CNS), said test animal being further characterized by being viable and fertile.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Transgenic Animals Lacking a Functional GFRα2 Receptor as well as Methods for their Production and Use

The Technical Field of the Invention

The present invention is related to transgenic non-human animals lacking a functional GFRα2 and their use as models for studying certain diseases as well as methods for producing cell-lines or clones lacking a functional GFRα2 receptor. The invention is also related to said cell lines, which are useful for producing the transgenic animals of the present invention.

The Background of the Invention

Glial cell line derived neurotrophic factor (GDNF) is a member of the transforming growth factor ß family. GDNF maintains the dopaminergic, noradrenergic, kolinergic and motoric nerve cells in the nerve system and also protects peripheric, parasympatic, siliar and sensoric nerve cells. During the development of mammals, GDNF is expressed also in many other tissues, such as embryonic kidney and testis. GDNF is related to neurturin NTN (Kotzbauer, P.T., et al., 1996). GDNF and NTN are known to have similar survival promoting activities on many central and peripheral neurons including motor, sympathetic, sensory and midbrain dopamine neurons (Lin, L.F., et al., 1993). Given these potent neurotrophic effects both *in vitro* and *in vivo*, it has been suggested that said GDNF and NTN and their receptors could play an important role in Parkinson's, Alzheimer's and motoneuron diseases. GDNF has been disclosed in WO 97/33912 and WO 98/52591 and NTN in WO 98/36072.

GDNF and NTN signal via multicomponent receptors that consist of the Ret receptor tyrosine kinase and one of the two structurally related GPI-linked receptors, GFRα1 and GFRα2, respectively (Jing, S., et al., 1996). Said receptors are also disclosed in WO 98/36072 and WO 98/52591. *In vitro* studies have suggested a high degree of interactions between the GDNF-family ligands and GFRα receptors. Although GDNF prefers GFRα1 and NTN prefers GFRα2, GDNF can activate Ret via GFRα2 and NTN can signal via GFRα1 (Baloh, R.H., et al. 1997; Buj-Bello, A., et al., 1997, Creedon, D.J., et al., 1997, Klein, R.D., et al., 1997; Sanicola, M., et al. 1997, Suvanto, P., et al., 1997). The terminology used is defined in GFRα nomenclature, 1997).

In developing and adult animals, GFRα receptors are abundantly expressed in various tissues,

i.e. their expression pattern is more widespread than that of the signaling receptor Ret (Trupp, M., et al., 1991, Widenfalk, J., et al., 1997) This suggests that the GFRα receptors may have roles independent of Ret. For example, GFRα2 is expressed in various regions of embryonic brain and spinal cord as well as in lung and bone, which show no or little expression of Ret. Thus, mice lacking these receptors were expected to show additional phenotypes than Ret and GDNF deficient mice, which die at birth due to lack of kidneys.

However, the present inventors surprisingly found that animals lacking functional GFRα receptors were viable and fertile but grew poorly and had an apparent ptosis. Ganglia from these mice lack neurite outgrowth responses to NTN, but normal responses to GDNF. The animals demonstrated specific deficits in autonomic, i.e. in enteric and parasympathetic, but not in sympathetic and sensory nervous system. Thus, the mice appeared useful as models for studying other diseases than those expected.

So far suitable models or test animals for studying gut disorders are lacking. However, there is a need to study diseases with unspecific symptoms caused by functional gut motility disorders, such as recurrent abdinomal pain and non-ulcer dyspepsia, which are quite common disorders, but so far lack adequate therapy and proper treatment modalities.

The transgenic animals of the present invention, which lack a functional GFRα2 provide a useful animal test model for studies and indicate that these mice may serve as models for several human diseases related, for example, to the malfunctions of gastrointestinal tract and certain autonomic and sensory neuropathies and epilepsy.

The Summary of the Invention

The characterstics of the present invention are as defined in the claims.

A Short Description of the Drawings

Figure 1 schematically depicts the construction of a targeting vector of the GFRα2 gene.

Figure 2 is a Southern blot of wild type and mutated allele of the GFRα2 gene.

Figure 3 is a photographic depiction of the expression of GFRα2 mRNA in wild type (+/+) and knockout (-/-) mouse brain *in situ* hybridization.

Figure 4 shows a Northern blot analysis of the expression of GFRα2 mRNA in wild type

(+/+) and knockout (-/-) mouse brain *in situ* hybridization.

Figure 5a shows the growth of female GFRα2 knockout mice and their littermate controls.

Figure 5b shows the growth of male GFRα2 knockout mice and their littermate controls.

Figure 6a is a photographic depiction of knockout mice.

Figure 6b is a photographic depiction of the littermate controls of the knockout mice.

Figure 7 shows a photographic depiction of AChE-histochemical staining of wild-type and mutant myenteric plexus.

Figure 8 shows the neurite outgrowth from E13 trigeminal ganglion explants of GFRα2 knockout mice. NTN+/+, NTN+/- and NTN-/- means, respectively, ganglia from wild-type, heterozygotous and homozygotous mutant mice, cultured in the presence of 5 ng/ml neurturin, GDNF+/+ and GDNF-/- means ganglia from wild-type and mutant mice, respectively, cultured in the presence of 5 ng/ml GDNF. No factor means that the ganglia were cultured in the medium without added throphic factors.

Figure 9 shows the quantification of neurite outgrowth response from E13 trigeminal ganglion explants of wild-type (+/+), heterozygotous (+/-) and homozygotous (-/-) mutant mice.

Figure 10 depicts the development of seizures during daily kindling stimulations of the hippocampus.

The Detailed Description of the Invention

Definitions

In the present invention the terms used have the meaning they generally have in the fields of biochemistry, pharmacology, neurology, recombinant DNA technology, including transgenic animal production related sciences, but some terms are used with a somewhat deviating or broader meaning than in the normal context. Accordingly, in order to avoid uncertainty caused by terms with unclear meaning some of the terms used in this specification and in the claims are defined in more detail below.

The term "**transgenic animal**" means any animals, with the exclusion of human beings, preferably produced by knock out techniques, but includes other *per se* known methods or methods developed in the future by which transgenic animals according to the present invention, lacking a functional GFRα2 receptor, can be produced. The term "**transgenic non-human animal**" includes especially animals useful as models for studying certain diseases and their remedies. Such animals are above all mice, rats, rabbits and hamsters.

The "**transgenic animals**" of the present invention are further characterized by having retarded postnatal growth, apparent ptosis, decreased nerve fiber density in the enteric nervous system, but they are viable and fertile.

The term "**apparent ptosis**" means that the animals prefer to keep their eyelids partially closed. This may be caused by weakness of the eyelid elevator muscles or by secondary reasons due to dry eyes, this may, for example, include increased blinking rate. The term "**apparent ptosis**" includes all types of ptosis including ptosis caused by secondary reasons.

The term "**viable and fertile**" means that the mutant mice show no significant increased lethality and can produce offspring of normal littersize.

The term "**retarded postnatal growth**" means that the mice are born normal sized but start to grow poorly during after weaning.

The term "**decreased nerve fiber density in enteric nervous system**" refers to reduced density of acetylcholinesterase (AChE)-positive nerve fibers in the myenteric plexus of the small intestine. These are mainly cholinergic exitatory fibers that innervate gut muscle wall, which results in the smooth muscle contraction and is the major cause of the enteric nervous system control of gut motility and rhythm.

The term "**model**" means test animal for developing therapies, studying and diagnosing disorders.

The term "**genetically modified compatible cell-line (clone)**" means cell-lines containing a genomic gene coding for a functional GFRα2 receptor.

The term "**functional GFRα2-receptor**" means that the genomic gene is functionally inactivated or conditionally inactivatable.

The term "**functionally inactivated**" means that the gene function is disrupted by mutation.

The term "**conditionally inactivatable**" means that the gene function can be inactivated only in certain tissues or at certain times.

The term "**developing therapies, studying and diagnosing disorders**" means especially growth related disorders, human sensory and autonomic nervous system disorders involving gut dysfunction and disorders in the specific peripheral nervous system (PNS), including autonomic and sensory functions as well as the in central nervous system (CNS).

The term "**sensory and autonomic nervous system disorders**" includes enteric and sympathetic, parasympatic and sensory disorders related to specific PNS (autonomic and sensory) functions. These include malfunctions of gastrointestinal tract (particularly its cholinergic, parasympathetic innervation) as well as of other cholinergic parasympathetic systems, e.g. lacrimal and salivary glands.

The term "**cell-line**" or "**clone**" means that the genomic gene encoding a functional GFRα2 receptor is inactivated by introducing into stem cells or cell lines (clones) a nucleic acid sequence or a part thereof, which sequence or part is capable of integrating with said genomic gene and by said integration functionally inactivates said genomic gene or makes it conditionally inactivatable.

The term "**nucleic acid sequence or a part thereof**" means such a nucleic acid sequence, which is homologous at least to a part of the gene encoding GFRα2 or to a part of the genome in the vicinity of said gene, to such a degree that it can hybridize with the single stranded gene and thus be integrated into the genome of the host animal and at by said integration the nucleic acid sequence acid functionally inactivates the gene.

A "**targeting vector**" means a DNA construct having a sufficient homology to allow hybridization with the gene or its vicinity. To isolate GFRα genomic clones, suitable animal libraries are screened e.g. with a rat GFRα2 cDNA fragment (Suvanto, P., et al., 1997) as a probe. Suitable fragments of the GFRα2 gene, containing for example part of the first coding exon with the translation initiation site, can be selected and inserted into a cassette in order to construct a targeting vector compatible with the host animal. A fragment from said cassette containing a selectable marker gene, e.g. a neomycin-resistance gene (neo^R gene) or another suitable marker gene driven by a suitable promoter and polyadenylation site, e.g. the PGK promoter and PGK polyadenylation signal (McBurney, M.W., et al., 1991) was selected as the targeting vector.

The term "**stem cells**" means the cells of the cell lines (clones) of the selected animal. Useful stem cells are embryonic stem cells (Nagy, A., et al., 1993), which can be cultivated on embryonic fibroblast feeder cells with leukemia inhibitory factor. The cells were electroporated with linearized plasmids and selected *per se* known methods. Resistant clones were screened by Southern blot analysis using a suitable external probe that recognizes a selected wild type and mutant band after a restriction enzyme digestion and could be identified at a reasonably high frequency, preferably at least 1/20, more preferably at least 1/30 most preferably at least 1/50. Additional integration of the vector was excluded by hybridization with the marker gene. Two injected clones gave germline transmission, when the chimeras were crossed to suitable wild type mice. If not otherwise stated, littermates from heterozygote matings were used for analysis.

The term "**marker genes**" means nucleic acid sequence capable of acting as markers, because they have a property, which enables them to be selected from a big population by screening, e.g. for their resistance of certain antibiotics or other materials, such as metals. Typical non-limiting examples of such markers are e.g. neomycin resistance or copper resistance gene.

The term "**GFRα2 cDNA fragment**" means the probe used for screening the selected library and which is obtainable from rat. However, it is evident that any other sufficiently similar fragment having at least a similarity of 70 %, preferably 80 %, most preferably at least 90 % at nucleotide level can be used.

The term "***In situ* hybridization**" means a histological techniques used for demonstrating the expression of specific mRNAs G from frozen tissue sections using suitable sense and antisense probes, synthesized using appropriate RNA polymerases. DNA templates were "**full-length**" rat GFRα2 (2018 bp of AF 003825 (Suvanto, P., et al., 1997)) and 5'-UTR mouse GFRα2 (424 bp of AA048808 (WashU-HHMI Mouse EST Project)).

The term "**ganglion explant cultivation**" means the magnitude of neurite outgrowth scored on a semi-quantitative scale. For the scoring animal trigeminal ganglia are prepared as described previously by Arumäe, U., et al., (1993) cultured and preferably in a three-dimensional matrix in the presence or absence of GDNF and NTN.

The term "**receptor**" means GDNF family binding substances, i.e. substances or compounds belonging to the GDNF family related compounds which are capable of transmitting the signals of GDNF family-related compounds. The term "**receptor**" above all means the GDNF signal remitting receptor cRet receptor tyrosine kinase.

The General Description of the Invention

Glial cell-line derived neurotrophic factor (GDNF) and a related protein, neurturin (NTN) show similar trophic effect on various cell populations. Their signaling via the Ret tyrosine kinase requires a ligand-binding, GPI-linked coreceptor, either GFRα1 or GFRα2, which are usually expressed in non-overlapping cell populations. *In vitro* studies suggest some promiscuity in their ligand specificities. To assess the *in vivo* function of GFRα2 and the physiological relevance of its ligand specificity, the present inventors have produced mutant mice lacking this receptor.

GFRα2 mutant mice appear normal at birth but become growth retarded compared to their wild type littermates postnatally. By age of one month, the mutants weigh approximately 30-50% less than the controls, and a significant difference in weight remains in adulthood. General reduction of organ size with proportional loss of adipose tissue suggests a specific systemic (possibly gastrointestinal or endocrine related) defect. Although gross anatomy of the intestine appears intact, preliminary results suggest less dense cholinergic fiber network in the myenteric plexus of duodenum which could result in functional defects. GFRα2 mutant mice are viable and fertile and lack obvious defects in muscle strength, motor activity or coordination. Expression of other GDNF family ligands and receptors including GDNF, NTN, GFRα1 and Ret were not altered in these mice as assessed by *in situ* hybridization (of E18 mouse embryos and adult brain.

Neurturin (5 ng/ml) induces a prominent neurite outgrowth from wild type embryonic trigeminal ganglion explants. In contrast, the neurturin response is virtually absent in mutant ganglia. In addition, less trigeminal neurons from mutant than from wild type embryos survive in presence of neurturin. In contrast, neuritic outgrowth and survival in response to GDNF (1-1000 ng/ml) is similar between the genotypes. Thus, GFRα2 does mediate neurturin responses *in vivo*, but is not required for GDNF responses. Since neurturin deficient mice have been reported to grow well, our results suggest that GFRα2 may also mediate *in vivo* biological responses of other factors, possibly other GDNF family members. The present inventors have shown that mice lacking functional GFRα2 coreceptor ($GFR\alpha2^{-/-}$) have ptosis and less dense innervation of small intestine. $GFR\alpha2^{-/-}$ mice are viable and fertile, but their growth becomes retarded after weaning probably due to malnutrition and disturbed gut motility. Hence, $GFR\alpha2^{-/-}$ mice may serve as a model for human gut motility disorders. Neuritogenesis and survival of $GFR\alpha2^{-/-}$ trigeminal neurons required 10-20 times higher NTN concentrations, suggesting signaling via GFRα1, whereas responses to GDNF were similar between the genotypes.

The transgenic non-human animals of the present invention are obtainable from a genetically modified compatible cell-line (clone) containing a genomic gene coding for a functional GFRα2 receptor, which genomic gene is functionally inactivated or conditionally inactivatable. The transgenic non-human animal of the present invention is especially useful as a model for developing therapies, studying and diagnosing growth related disorders, human sensory and autonomic nervous system disorders involving gut dysfunction and disorders in the specific peripheral nervous system (PNS), including autonomic and sensory functions as well as the in central nervous system (CNS). Said test animal are further characterized by being viable and fertile and having at least one of the following properties. They have a retarded postnatal growth, ptosis and/or decreased nerve fiber density in the enteric nervous system. The present invention is also related to a methods for obtaining a cell-lines or clones, which can be used as a source for producing transgenic non-human animals.

In the method of the present invention, the genomic gene encoding a functional GFRα2 receptor is inactivated by introducing into stem cells or cell lines (clones) a nucleic acid sequence or a part thereof, which sequence or part is capable of integrating to said genomic gene and by said integration functionally inactivates said genomic gene or makes it conditionally inactivatable.

The cell-lines or clone lacking a functional GFRα2 receptor are obtainable for example by a method comprising the steps described below.

The desired nucleic acid sequence or a part thereof, can be obtained by screening a selected library by using as a probe a known GFRα2 cDNA fragment, obtained from rat. The selected nucleic acid sequence obtained by screening is capable of being integrated into the genomic gene coding for a functional GFRα2 receptor. The integration functionally inactivates the genomic gene or makes it conditionally inactivatable.

Targeting constructs can be prepared by combining the nucleic acid sequence or a part thereof with a selectable nucleic acid sequence acting as a marker or by preparing a targeting vector by inserting the nucleic acid sequence or a part thereof into a cassette containing a selectable nucleic acid sequence capable of acting as a marker. The selectable nucleic acid sequence acting as a marker can be but is by no means limited to the neomycin resistance gene.

When the targeting construct or vector is introduced into stem cells or cell lines (clones) of

the selected animal and the cell lines (clones) now containing the desired nucleic acid sequence or part thereof, which can be integrated into the genomic gene of said stem cell, Those cell lines (clones), into which the targeting construct or vector has been incorporated can be selected using a probe, which is capable of recognizing the selectable marker nucleic acid sequence. Thus, the invention is also related to cell lines or clones obtainable by the method described above or similar methods. The cell-lines or clones are lacking a functional GFRα2 receptor.

The cell lines of the present invention are used for producing transgenic non-human animals, which are useful as test animals or models for developing therapies, studying and diagnosing growth related disorders, human sensory and autonomic nervous system disorders involving gut dysfunction and disorders in the specific peripheral nervous system (PNS), including autonomic and sensory functions as well as in central nervous system (CNS).

The cell lines or clones carrying a functionally inactivated or conditionally inactivatable genomic gene are incorporated into the selected animal using *per se* known methods. Thus, the ultimate goal of the present invention is to provide a novel method for assessing *in vivo* functions, developing therapies, studying and diagnosing growth related disorders and human sensory and autonomic nervous system disorders involving gut dysfunction. The disorders are assessed, studied and diagnosed and cures are developed by the aid of the selected transgenic animal lacking a functional GFRα2 receptor described in the present invention.

Even if the applicability and utility of the present invention has been demonstrated with mice, it is evident that similar animals can be produced by adapting to them the method described herein. As a conclusion, it has been shown that mice lacking GFRα2 have postnatal growth retardation and mild ptosis, but are fertile and viable. They are characterized by a general reduction in organ size with proportional decrease of fat tissue suggests a specific systemic defect, which is possibly endocrine related and/or gastrointestinal. This may also be related to the impairment in gastrointestinal innervation, since a reduction in the density of cholinergic fiber network in the gastrointestinal tract and in particular in the myoenteric plexus of the duodenum of the mutant mice has been demonstrated. Neurite outgrowth assays show that GFRα2 is the receptor for neurturin (NTN) *in vivo*, but not for GDNF at physiological concentrations. Together with the known expression of GFRα2 in sensory ganglia (Luukko, K., et al., 1997; Naveilhan, P., et al., 1997; Bennett, D.L.H., et al., 1998), the results disclosed in the present invention indicate that NTN plays a role in specific sensory neurons. Furthermore, the growth phenotype suggests that GFRα2 may also mediate biological effects of other growth factors than neurturin. Experiments with hippocampal kindling show that GFRα2 can modulate susceptibility to seizures.

Results of the analysis of the mutant mice lacking GDNF family receptor α2 indicated that these mice may serve as the model for several human diseases, especially such related to the malfunctions of gastrointestinal tract and other organs innervated by GFRα-responsible autonomic and sensory neurons. Changes in the GFRα2 expression levels in the brain in the rat models of epilepsy and ischemia, and the resistance of the GFRα2-deficient mice to kindling seizure indicate that the GFRα2 mutant mice may serve as models of epilepsy, ischemia and related disorders. High expression levels of GFRα2 in the developing cartilage and bone, as well as changes in the GFRα2 levels in the brains of rats in rat models of epilepsy and ischemia indicate that GFRα2 mutant mice may serve also as models for the epilepsy, ischemia and for diseases related to cartilage/bone development. Even if the results are shown in mice only, it is obvious to one skilled in the art that the same or similar results could be obtained with other animals, but would require additional experiments with said other animals.

The invention is described in more detail in the following experimental part, which discloses the material and methods used for the preparation of transgenic mice and the experiments used to demonstrate the effect of GDNF and NTN in the animal model. Said experimental part should not be construed to limit the scope of the invention. Those skilled in the art can foresee a multitude of different application based on the results obtained in the following experimental part.

Example 1
Isolation of genomic clones and gene targeting

To isolate GFRα2 genomic clones, a mouse 129/Sv library (Stratagene) was screened with a rat GFRα2 cDNA fragment (Suvanto, P., et al., 1997) as a probe. A 6.7 kb HindIII - XbaI fragment was used to construct the targeting vector **(Figure 1)**. A 0.5 kb NotI-XbaI fragment of the GFRα2 gene, containing part of the first coding exon with the translation initiation site, was replaced with a 2.0 kb cassette containing the neomycin-resistance gene (neo^R gene) driven by the PGK promoter and PGK polyadenylation signal (McBurney, M.W., et al., 1991).

R1 embryonic stem cells (Nagy, A., et al., 1993), grown on embryonic fibroblast feeder cells with leukemia inhibitory factor, were electroporated with linearized plasmid and selected in G418 (250μg/ml). Resistant clones were screened by Southern blot analysis using an external probe that recognizes a 7.8 kb wild type and 5.5 kb mutant band after a BamHI digest and were identified at 1/50 frequency. Additional integration of the vector was

excluded by hybridization with a neo probe (not shown). Two injected clones gave germline transmission, when the chimeras were crossed to C57BL/6 or 129Sv wild type mice. If not otherwise stated, littermates from heterozygote matings were used for analysis.

Example 2.
Southern blot analysis of tails

Animals were genotyped by Southern blot with an 5' outside probe (indicated in **(Figure 1)** illustrating the 9 kb wild type and 3.6 kb targeted allele hybridizing bands following BamHI digestion **(Figure 2).**

Example 3
***In situ* hybridization depicting expression of GFRα mRNA in brain**

Expression of GFRα2 mRNA was tested in the brains of the adult wild type and mutant mice, lacking GFRα2 using *in situ* hybridization **(Figure 3)** and by Northern blotting **(Figure 4)**. The results of the *in situ* hybridization reveal a strong expression of the GFRα2 mRNA in the cortex, hippocampus, reticular thalamic nucleus, medial habenular nucleus, zona inserta etc. In the mutant mice, where the GFRα2 gene has been disrupted a low level expression of the GFRα2-like mRNA can be detected exactly in the same brain regions as in wild type littermate control mice. This result shows that detection of the GFRα2-like mRNA allows to identify the cells survived in the mutant mice, but expressing GFRα2 in wild type mice. Northern blotting analysis demonstrates that the mRNA for normal GFRα2 is 4 kb long, whereas in mutant mice only the truncated mRNA of about 0.8 kb can be detected.

Example 4.
Poor growth and apparent ptosis in the mutant mice

A. Growth of the mutant mice and their littermate controls was followed from the moment of birth for 80 days by measuring their weight. Female **(Figure 5a)** and male mice **(Figure 5b)** were analyzed separately. The results demonstrate that during the first 15-20 postnatal days the growth of mutant and their littermate control mice is normal - there is no difference in their weight. However, starting from the day 15-20 the growth of the GFRα2 mutant mice is retarded, being very manifested at the age of 1 months, when the difference in weight can be up to 30-50% compared to the controls. The mutant mice grow slower also during the following 2 postnatal months and at the age of 3 months the mutant mice are approximately 30% smaller than their littermate controls. It should be noted that the growth retardation of

the male mice is somewhat more pronounced. These results show that GFRα2 mutant mice have a growth retardation phenotype.

B. Mutant mice at the age of one month are about 50 % smaller than their littermate controls **(Figure 6a)** and show apparent ptosis **(Figure 6b)**. The apparent ptosis can be due to a muscle problem in lid elevation or be a secondary symptom caused by dry eye and lacrimal gland dysfunction. Our subsequent studies revealed that the reason for the apparent ptosis is most likely the lack of parasympathetic innervation of lacrimal gland (Rossi, et al., 1999).

Example 5.
Staining of duodenum

One month old wild type (+/+) and homozygous mutant (-/-) littermate animals were perfused with 4% paraformaldehyde. Acetylcholinesterase (AChE) staining of whole mount duodenum was according to Tago, H., et al., (1986), but 1% Triton X-100 was included in the staining solution to increase penetration. The density of fine AChE-positive fiber plexus is clearly reduced in the mutant small intestine compared to its wild-type littermate controls **(Figure 7)**.

Example 6.
Neurite outgrowth from ganglion explant cultures.

E13 and E15 mouse trigeminal ganglia were prepared as described previously (Arumäe, U., et al., 1993). Trigeminal ganglia were cultured in three-dimensional collagen matrix as described by Ebendal (1989). Recombinant GDNF and NTN proteins purchased from PreproTech, and GDNF donated by Cephalon, Inc. were also used, and applied at concentrations ranging from 1 to 100 ng/ml. For controls, trigeminal ganglia were cultured without GDNF and NTN. The explants were cultured in a humidified atmosphere of 5% CO_2 in air at +37 °C for 48 hr. The magnitude of neurite outgrowth was scored on a semi-quantitative scale.

Neurite outgrowth from embryonic trigeminal ganglion of GFRα2 mutant and wild type littermate control mice explants at embryonic day E13 was tested in the presence of GDNF and NTN and the results are shown in **Figure 8**. $NTN^{+/+}$ induces a neuritic halo from the ganglionic explants of wild type mice, where no neurite outgrowth can be detected when E13 trigeminal ganglion explants from the GFRα2 mutant mice were tested $NTN^{-/-}$. Interestingly, NTN induces a visible neurite outgrowth from ganglionic explants, prepared from the GFRα2 heterozygous mice ($NTN^{+/-}$). In contract, GDNF promotes an extensive

neurite outgrowth from the trigeminal ganglia explants of GFRα2 mutant mice GDNF$^{+/-}$, which is indistinguishable from that of the wild type mice GDNF$^{+/+}$. Control experiments clearly demonstrate that growth media has no neurite outgrowth-promoting activity NO FACTOR$^{+/+}$. These results clearly show that GFRα2 mediates NTN responses *in vivo*, but GDNF does not. Moreover, since NTN can promote neurite outgrowth from trigeminal ganglia of heterozygous animals, it is possible that GFRα2 may act as a growth factor.

Quantification of the neurite outgrowth from E13 trigeminal ganglion explants of GFRα2 knockout mice. wt- trigeminal ganglion explant cultures were prepared from the embryos of wild type mice and grown in the presence of 5ng/ml of GDNF, or 5 ng/ml of NTN or without added factor. Similar experiments were carried out with E13 trigeminal ganglion explants prepared from mutant mice (-/-) and from heterozygous mice (+/-). The quantification was performed by measuring the average fiber length and density. The results of these assays support and extend the conclusions drawn in **Figure 9** and further support the notion that NTN signaling in vivo is mediated by GFRα2 receptor.

Example 7

Suppressed epileptogenesis in GFRα mutant mice

Kindling-induced seizures lead to region- and cell-specific changes of mRNAs for GDNF, NTN and their receptors in the adult rat brain. In order to test the possibility that the changes in endogenous levels of NTN and their receptors following seizures may influence the development of epileptic syndromes, GFRα2-deficient mice (GFRα2-/-) were subjected to daily stimulations according to the conventional hippocampal kindling paradigm. The results are shown in **Figure 10**, which shows the grading of seizures during development of hippocampal kindling (gr 0=no seizures to gr 5 = full-blown tonic-clonic convulsions). +/+ = wild-type littermate controls. From the results it can be concluded that the development of kindling was markedly suppressed in mice lacking GFRα2. These animals did not reach the fully kindled state (3 grade 5 seizures) until after 38±4 days of stimulation as compared t0 21±2days in the wild-type mice. Test stimulations performed 4-6 weeks after the fifth grade 5 seizure, revealed that increased excitability was maintained in GFRα2-/-mice, but the magnitude was lower as compared to wild-type mice. The result suggest that modulation of GFRα2 mediated signaling may be useful in treatment of epilepsy.

References

Arumäe, U., Pirvola, U., Kiema, T.-R., Palm, K., Moshnyakov, M., Ylikoski, J. and Saarma, M. (1993) Neurotrophins and their receptors in rat peripheral trigeminal system during maxillary nerve growth. J Cell Biol 122, 1053-1065.

Baloh, R.H., Tansey, M.G., Golden, J.P., Creedon, D.J., Heuckeroth, R.O., Keck, C.L., Zimonjic, D.B., Popescu, N.C., Johnson, E.M., Jr., and Milbrandt, J. (1997). TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret. Neuron 18, 793-802.

Bennett, D.L.H., Michael, G.J., Ramachandran, N., Munson, J.B., Averill, S., Yan, Q., McMahon, S.B., and Priestley, J.V. (1998). A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. J Neurosci. 18, 3059-3072.

Buj-Bello, A., Adu, J., Pinon, L.G., Horton, A., Thompson, J., Rosenthal, A., Chinchetru, M., Buchman, V.L., and Davies, A.M. (1997). Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. Nature 387, 721-724.

Creedon, D.J., Tansey, M.G., Baloh, R.H., Osborne, P.A., Lampe, P.A., Fahrner, T.J., Heuckeroth, R.O., Milbrandt, J., and Johnson, E.M., Jr. (1997). Neurturin shares receptors and signal transduction pathways with glial cell line-derived neurotrophic factor in sympathetic neurons. Proc Natl Acad Sci U S A 94, 7018-7023.

Ebendal, T. (1989) Use of collagen gels to bioassay nerve growth factor activity. In: Rush RA, Ed. Nerve growthj fators. Chichester: John Wiley and Sons Ltd. 81-93.

Heuckeroth, R.O., Tourtellotte L., Gavrilina, G., Johnson, E., and Milbrandt J. Characterization of the neurturin knockout mouse. Soc. Neurosci. Abstr. Vol. 23, Part 2, p. 1704, 1997.

Heinicke, E.A., Kiernan J.A., and Wijsman J. (1987) Specific, selective and complete staining of neurons of the myenteric plexus, using Cuprolinic blue. J. Neurosci. Meth. 21, 45-54.

Hunt S.P., Mantyh P.W. and Priestley J.V. The organization of biochemically characterized

sensory neurons. In (Scott S.A., ed.): Sensory Neurons, Diversity, Development and Plasticity, pp. 60-76. Oxford University Press (1992).

Jing, S., Wen, D., Yu, Y., Holst, P.L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., Louis, J.C., Hu, S., Altrock, B.W., and Fox, G.M. (1996). GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. Cell 85, 1113-1124.

Jing, S., Yu, Y., Fang, M., Hu, Z., Holst, P.L., Boone, T., Delaney, J., Schultz, H., Zhou, R., and Fox, G.M. (1997). GFRalpha-2 and GFRalpha-3 are two new receptors for ligands of the GDNF family. J Biol Chem 272, 33111-33117.

Kallunki P, Sainio K, Eddy R, Byers M, Kallunki T, Sariola H, Beck K, Hirvonen H, Shows TB, Tryggvason K. (1992) A truncated laminin chain homologous to the B2 chain: structure, spatial expression, and chromosomal assignment. J Cell Biol. 119, 679-693.

Klein, R.D., Sherman, D., Ho, W.H., Stone, D., Bennett, G.L., Moffat, B., Vandlen, R., Simmons, L., Gu, Q., Hongo, J.A., Devaux, B., Poulsen, K., Armanini, M., Nozaki, C., Asai, N., Goddard, A., Phillips, H., Henderson, C.E., Takahashi, M., and Rosenthal, A. (1997). A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature 387, 717-721.

Kotzbauer, P.T., Lampe, P.A., Heuckeroth, R.O., Golden, J.P., Creedon, D.J., Johnson, E.M., Jr., and Milbrandt, J. (1996). Neurturin, a relative of glial-cell-line-derived neurotrophic factor. Nature 384, 467-470.

Lin, L.F., Doherty, D.H., Lile, J.D., Bektesh, S., and Collins, F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons [see comments]. Science 260, 1130-1132.

Luukko, K., Suvanto, P., Saarma, M., and Thesleff, I. (1997). Expression of GDNF and its receptors in developing tooth is developmentally regulated and suggests multiple roles in innervation and organogenesis. Dev Dyn 210, 463-471.

McBurney, M.W., Sutherland, L.C., Adra, C.N., Leclair, B., Rudnicki, M.A., Jardine, K. (1991) The mouse Pkg-1 gene promoter contains an upstream activator sequence. Nucl. Acids Res. 19, 5755-5761.

Milbrandt, J., de Sauvage, F.J., Fahrner, T.J., Baloh, R.H., Leitner, M.L., Tansey, M.G., Lampe, P.A., Heuckeroth, R.O., Kotzbauer, P.T., Simburger, K.S., Golden, J.P., Davies, J.A., Vejsada, R., Kato, A.C., Hynes, M., Sherman, D., Nishimura, M., Wang, L.C., Vandlen, R., Moffat, B., Klein, R.D., Poulsen, K., Gray, C., Garces, A., and Johnson, E.M., Jr. (1998). Persephin, a novel neurotrophic factor related to GDNF and neurturin. Neuron 20, 245-253.

Molliver, D.C., Wright, D.E., Leitner, M.L., Parsadanian, A.S., Doster, K., Wen, D., Yan, Q., and Snider, W.D. (1997). IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. Neuron 19, 849-861.

Nagy, A., Rossant, J., Nagy, R., Abrahamow-Newrly, W., and Roder, J.C. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc. Natl.Acad.Sci. USA 90, 8424-8428.

Naveilhan, P., Baudet, C., Mikaels, A., Shen, L., Westphal, H., and Ernfors, P. (1998). Expression and regulation of GFRalpha3, a glial cell line-derived neurotrophic factor family receptor. Proc Natl Acad Sci U S A 95, 1295-1300.

Pachnis, V., Mankoo, B., and Costantini, F. (1993). Expression of the c-ret proto-oncogene during mouse embryogenesis. Development 119, 1005-1017.

Rossi, J., Luukko, K., Poteryaev, D., Laurikainen, A., Sun, Y.-F., Laakso, T., Eerikäinen, S., Tuominen, R. Laakso, M., Rauvala, H., ArumÄe, U., Saarma, M. and Airaksinen, M.S. (1999). Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFRα2, a functional neurturin receptor. Neuron 22:243-252.

Sainio, K., Suvanto, P., Davies, J., Wartiovaara, J., Wartiovaara, K., Saarma, M., Arumae, U., Meng, X., Lindahl, M., Pachnis, V., and Sariola, H. (1997). Glial-cell-line-derived neurotrophic factor is required for bud initiation from ureteric epithelium. Development 124, 4077-4087.

Sanicola, M., Hession, C., Worley, D., Carmillo, P., Ehrenfels, C., Walus, L., Robinson, S., Jaworski, G., Wei, H., Tizard, R., Whitty, A., Pepinsky, R.B., and Cate, R.L. (1997). Glial cell line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins. Proc Natl Acad Sci U S A 94, 6238-6243.

Suvanto, P., Hiltunen, J.O., Arumae, U., Moshnyakov, M., Sariola, H., Sainio, K., and

Saarma, M. (1996). Localization of glial cell line-derived neurotrophic factor (GDNF) mRNA in embryonic rat by in situ hybridization. Eur. J Neurosci. 8, 816-822.

Suvanto, P., Wartiovaara, K., Lindahl, M., Arumae, U., Moshnyakov, M., Horelli-Kuitunen, N., Airaksinen, M.S., Palotie, A., Sariola, H., and Saarma, M. (1997). Cloning, mRNA distribution and chromosomal localisation of the gene for glial cell line-derived neurotrophic factor receptor beta, a homologue to GDNFR-alpha. Hum Mol. Genet 6, 1267-1273.

Tago H, Kimura H, and Maeda T. (1986) Visualization of detailed acetylcholinesterase fiber and neuron staining in rat brain by a sensitive histochemical procedure. J. Histochem. Cytochem. 34, 1431-1438.

Trupp, M., Arenas, E., Fainzilber, M., Nilsson, A.S., Sieber, B.A., Grigoriou, M., Kilkenny, C., Salazar-Grueso, E., Pachnis, V., and Arumae, U. (1996). Functional receptor for GDNF encoded by the c-ret proto-oncogene [see comments]. Nature 381, 785-788.

Trupp, M., Belluardo, N., Funakoshi, H., and Ibanez, C.F. (1997). Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor- alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. J Neurosci. 17, 3554-3567.

Widenfalk, J., Nosrat, C., Tomac, A., Westphal, H., Hoffer, B., and Olson, L. (1997). Neurturin and glial cell line-derived neurotrophic factor receptor-beta (GDNFR-beta), novel proteins related to GDNF and GDNFR-alpha with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. J Neurosci. 17, 8506-8519.

GFR(alpha) Nomenclature Committee. Nomenclature of GPI-linked receptors for the GDNF ligand family. Neuron 19: 485 (1997).

Claims

1. A transgenic non-human animal, **characterized** in that said animal is lacking a functional GFRα2 receptor and is useful as a test animal.

2. The transgenic non-human animal of claim 1, **characterized** in that said animal is obtainable from a genetically modified compatible cell-line (clone) containing a genomic gene coding for a functional GFRα2 receptor, which genomic gene is functionally inactivated or conditionally inactivatable.

3. The transgenic non-human animal of claims 1-2, **characterized** in that said animal is useful as a test animal or model for developing therapies, studying and diagnosing growth related disorders, human sensory and autonomic nervous system disorders involving gut dysfunction and disorders in the specific peripheral nervous system (PNS), including autonomic and sensory functions as well as the in central nervous system (CNS), said test animal being further characterized by being viable and fertile and having at least one of the following properties
(a) retarded postnatal growth;
(b) apparent ptosis;
(c) decreased nerve fiber density in the enteric nervous system.

4. The transgenic non-human animal of claims 1-3, **characterized** in that it is a mouse.

5. A method for obtaining a cell-line (clone) useful in the production of the transgenic non-human animal according to claims 1-4, **characterized** in that the genomic gene encoding a functional GFRα2 receptor is inactivated by introducing into stem cells or cell lines (clones) a nucleic acid sequence or a part thereof, which sequence or part is capable of integrating to said genomic gene and by said integration functionally inactivates said genomic gene or makes it conditionally inactivatable.

6. The method of claim 5, **characterized** in that the cell-line (clone) lacking a functional GFRα2 receptor is obtainable by a method comprising the following steps:

(a) providing a nucleic acid sequence or a part thereof, which sequence or part thereof is capable of being integrated into the genomic gene coding for a functional GFRα2 receptor and by said integration functionally inactivates the genomic gene or makes it conditionally inactivatable, said nucleic acid sequence or part thereof being obtainable by screening a

selected library, using a known GFRα2 cDNA fragment as a probe;
(b) preparing a targeting construct by combining the nucleic acid sequence or a part thereof according to step (a), with a selectable nucleic acid sequence acting as a marker; or
preparing a targeting vector by inserting at a nucleic acid sequence or a part thereof according to step (a) into a cassette containing a selectable nucleic acid sequence acting as a marker;
(c) introducing the targeting construct or vector of step (b) into stem cells or cell lines (clones) of the selected animal;
(d) selecting a cell line (clone) containing the nucleic acid sequence or part thereof according to step (a) which has been integrated into the genomic gene of said stem cell or cell line (clone) using a probe, which is capable of recognizing the selectable nucleic acid sequence acting as a marker.

7. The method of claim 6, **characterized** in that the known GFRα2 cDNA fragment used as a probe for screening the selected library is obtainable from rat.

8. The method of claim 6, **characterized** in that the probe which is capable of recognizing the selectable nucleic acid sequence acting as a marker is a neomycin resistance gene.

9. A cell line (clone) obtainable by the method of claims 5-8, **characterized** in that the cell-line (clone) is lacking a functional GFRα2 receptor.

10. The use of the cell line (clone) of claim 9, **characterized** in that the cell lines are used for producing a transgenic non-human animal useful as a test animal or model for developing therapies, studying and diagnosing growth related disorders, human sensory and autonomic nervous system disorders involving gut dysfunction and disorders in the specific peripheral nervous system (PNS), including autonomic and sensory functions well as in central nervous system (CNS) by introducing the cell line (clone) carrying a functionally inactivated or conditionally inactivatable genomic gene into the selected animal using *per se* known methods.

11. A method for assessing *in vivo* functions, developing therapies, studying and diagnosing growth related disorders and human sensory and autonomic nervous system disorders involving gut dysfunction, **characterized** in that the disorders are assessed, studied and diagnosed and cures are developed by the aid of the selected transgenic animal lacking a functional GFRα2 receptor useful as a test animal.

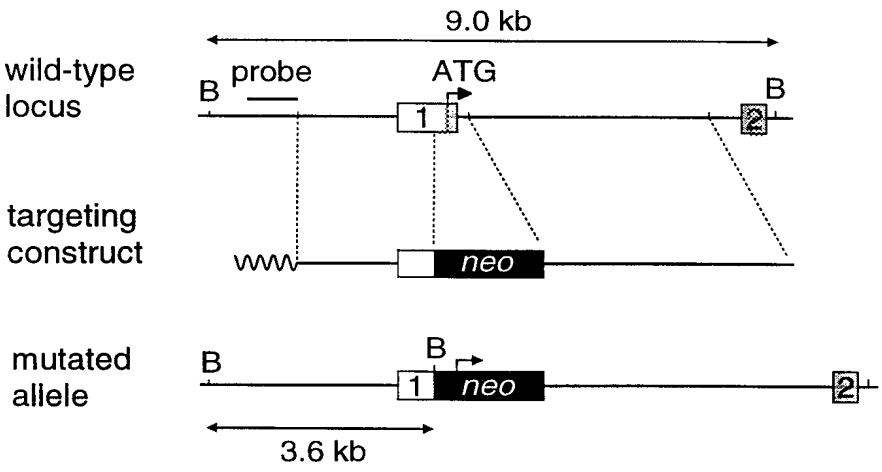

9.0 kb
wild-type locus
B
probe
ATG
1
2
B
targeting construct
neo
mutated allele
B
B
1
neo
2
3.6 kb

Fig. 1

Southern blot

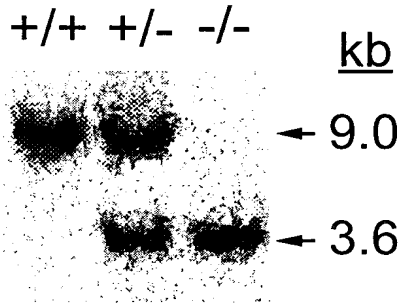


Fig. 2

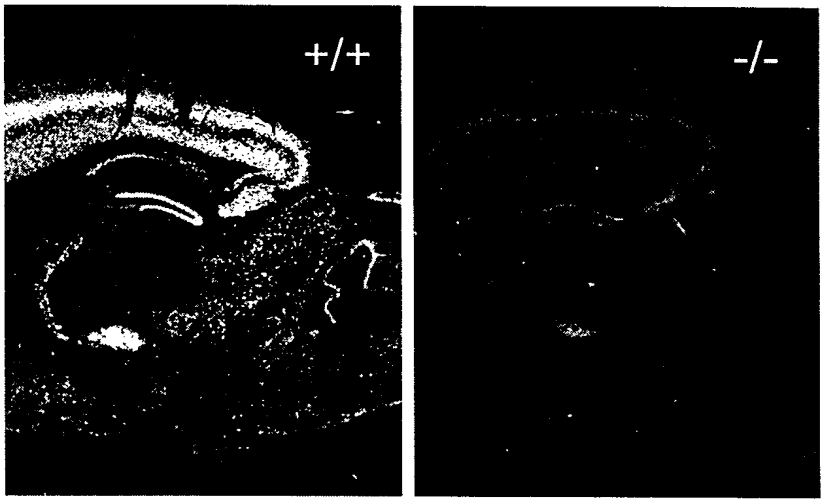


Fig. 3

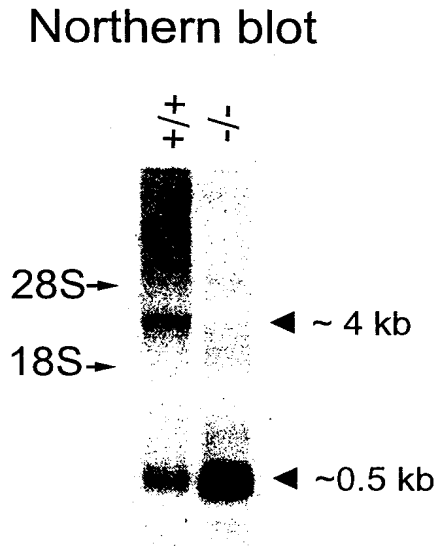


Fig. 4

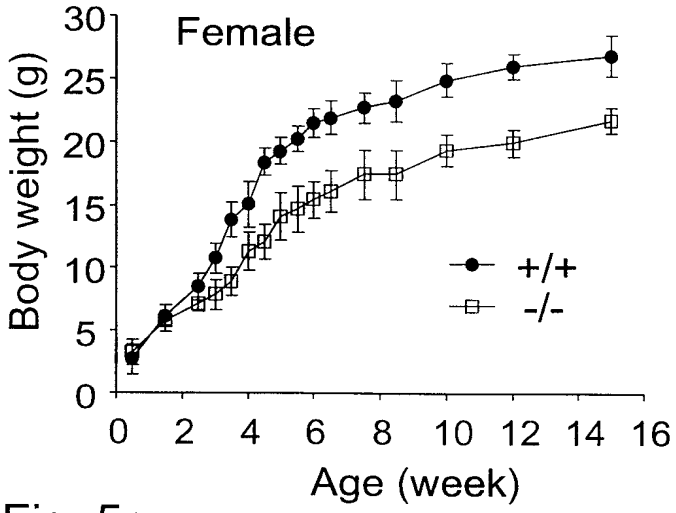

Female
Body weight (g)
30
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16
Age (week)
+/+
-/-

Fig. 5a

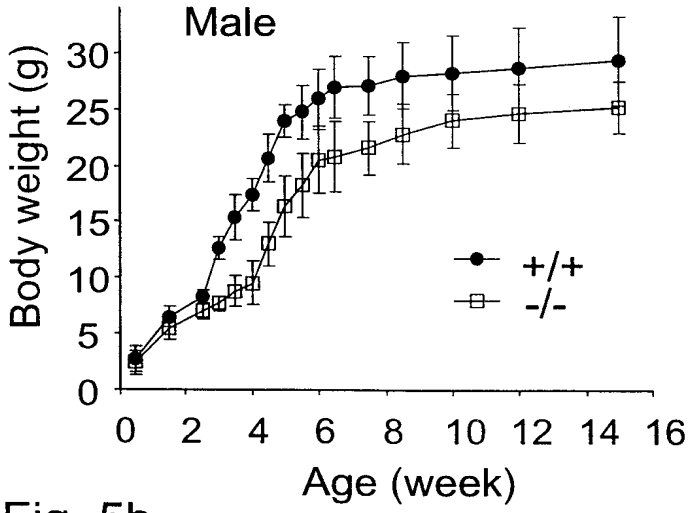
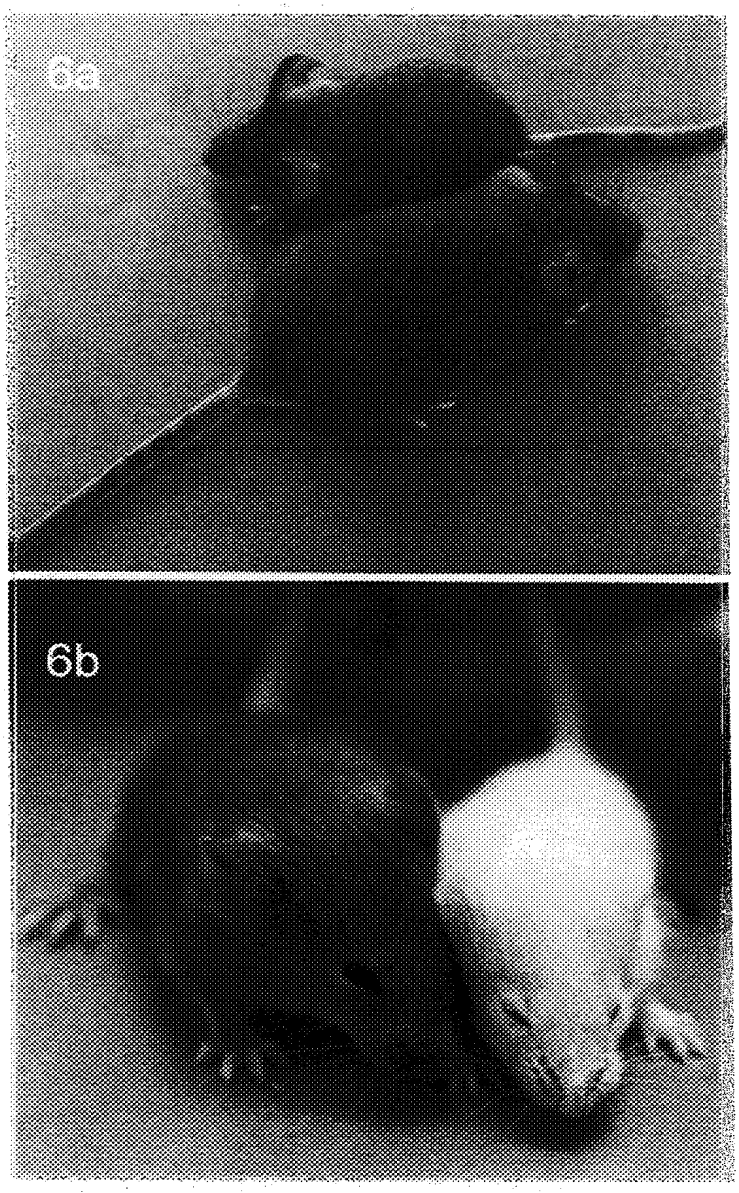

Male
Body weight (g)
30
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16
Age (week)
+/+
-/-

Fig. 5b



Gfra2 knockout mice
and their littermates

Fig. 6

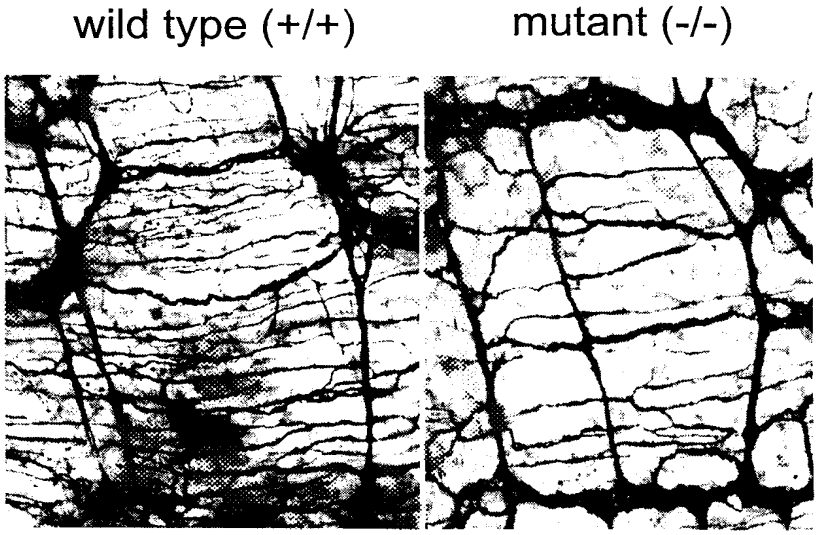


Fig. 7

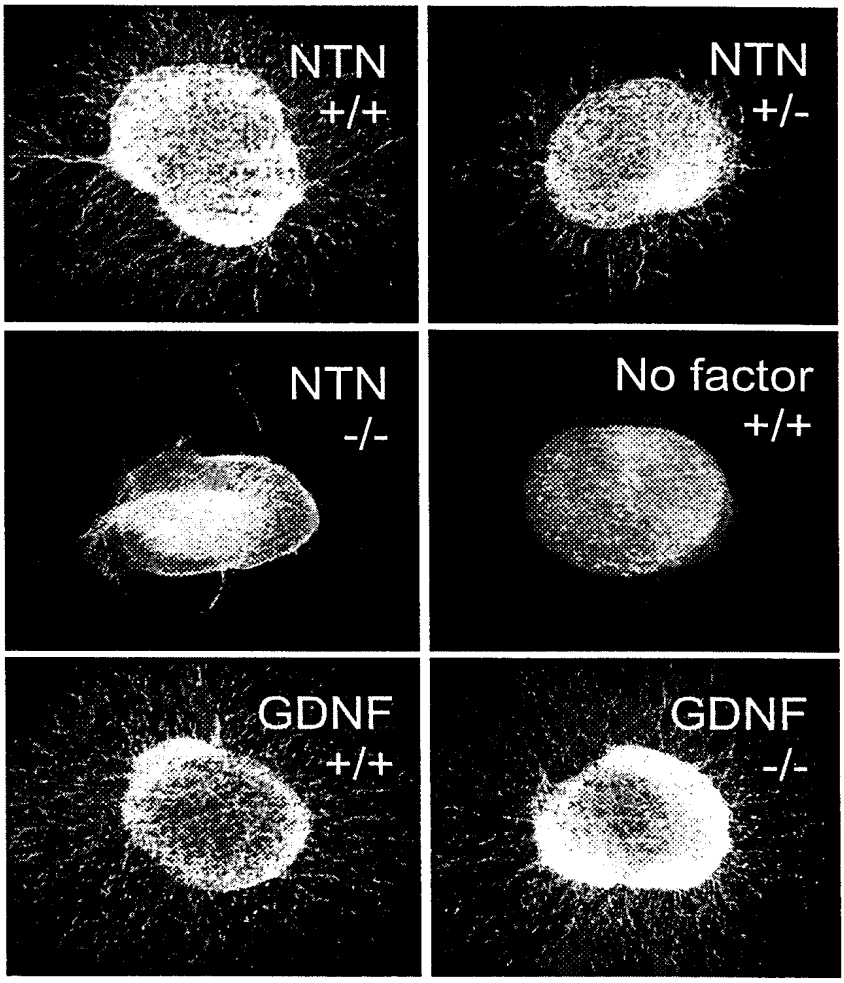


Fig. 8

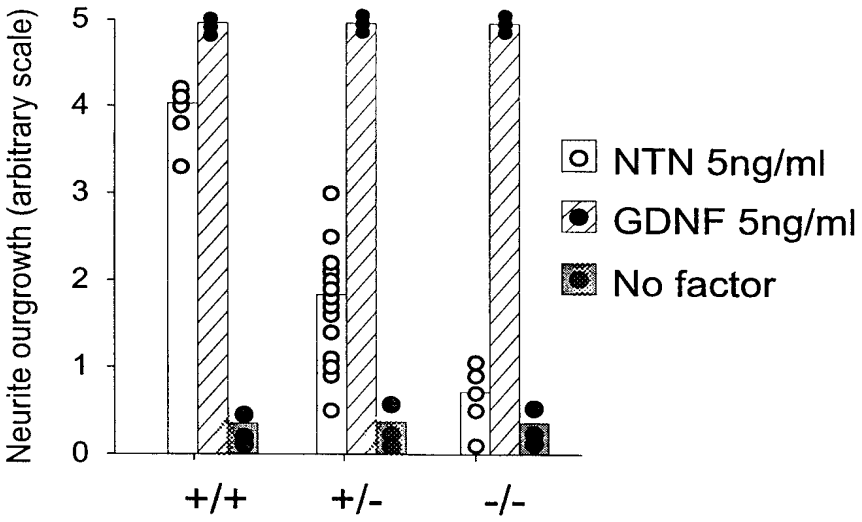

Neurite ourgrowth (arbitrary scale)
0
1
2
3
4
5
+/+
+/-
-/-
NTN 5ng/ml
GDNF 5ng/ml
No factor

Fig. 9

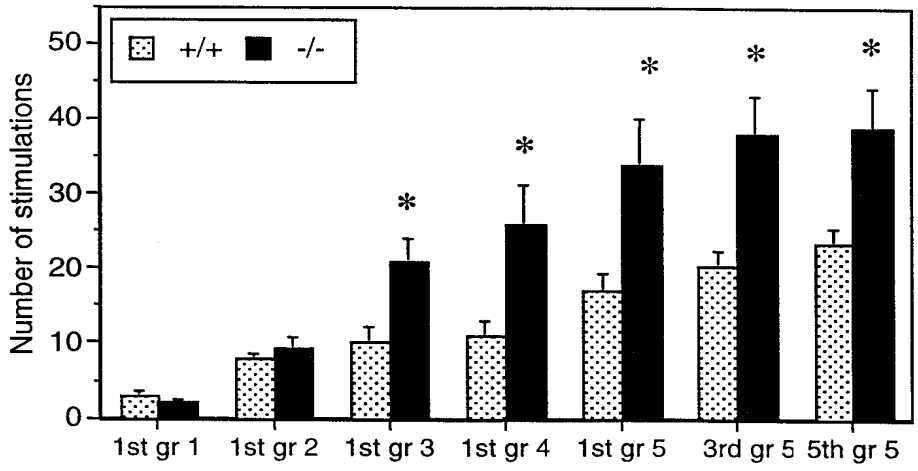

+/+
-/-
Number of stimulations
0
10
20
30
40
50
1st gr 1
1st gr 2
1st gr 3
1st gr 4
1st gr 5
3rd gr 5
5th gr 5
*
*
*
*
*

Fig. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 99/00464

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A01K 67/027, C12N 5/16 // C 07 K 14/71
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A01K, C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JBC Online, Volume 272, No 52, December 1997, S. Jing et al., "GFRalfa-2 and GFRalfa-3 Are Two New Receptors for Ligands of the GDNF Family", pages 33111-33117 --	1-11
P,X	Neuron, Volume 22, No 2, February 1999, J. Rossi et al., "Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFR alpha2, a functional neurturin receptor" page 243 - page 252 -- --------	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 Sept 1999	07 -09- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)